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TITLE:
Targeting Common but Complex Proteoglycans on Breast Cancer Cells and Stem Cells Using Evolutionary Refined Malaria Proteins

PRINCIPAL INVESTIGATOR: **Mads Daugaard**

CONTRACTING ORGANIZATION: **British Columbia Cancer Agency Branch**
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13. SUPPLEMENTARY NOTES

(*) On December 1st, 2014 Dr. Mads Daugaard relocated from BC Cancer Agency (BCCA) to the Vancouver Prostate Centre (VPC), Faculty of Medicine, University of British Columbia (UBC), Vancouver, BC. A request for transferring Dr. Daugaard's portion of the award from BCCA to VPC/UBC has been submitted and is under consideration by USAMRAA Contract Specialist Cheryl A. Lowery.

14. ABSTRACT

In the past year of the project we have successfully produced recombinant VAR2CSA that binds with high affinity and high specificity to breast cancer cells and breast cancer tissue biopsies. We have demonstrated that the protein binds to distinct secondary modified proteoglycans exclusively present on breast cancer cells and that targeting these with recombinant VAR2CSA interferes with key functions of the cancer cell like growth, migration and invasion.

15. SUBJECT TERMS

Cancer, breast cancer, treatment, malaria protein

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Assistant Professor Mads Daugaard
BC Cancer Agency*

Award in Transfer:

*Previous organization.

**New organization: Dr. Daugaard relocated in Dec 2014 from BCCA to Vancouver Prostate Centre (VPC), University of British Columbia. The transfer request and BCCA relinquishment statement was submitted on November 14th, 2014.

USAMRA Activity contract specialist **Cheryl A. Lowery** is processing transfer of Dr. Daugaard's portion of the grant.

1. INTRODUCTION:

In pregnant women, parasite-infected red blood cells express a protein that binds to a distinct sugar structure present only on certain cells of the placenta. This highly evolved binding system enables the parasite to evade clearance and infect placental cells, thereby causing pregnancy-associated malaria outbreaks in epidemic regions of the world. Prior to this application we discovered that human breast cancer cells express this same carbohydrate structure, which is present on human placental cells. The sugar structure likely enables breast cancer cells to migrate and invade surrounding normal tissue, and plays a role in metastatic spread of the cancer. This raised the intriguing possibility that we could use this naturally refined parasite-host interaction mechanism as a tool to specifically bind human breast cancer cells and inhibit their metastatic potential. The aim of the project was to combine our individual expertise in parasitology (Dr. Salanti) and oncology (Drs. Daugaard and Sorensen) to investigate the potential of exploiting the interaction between the malarial protein, called VAR2, and the breast cancer-associated sugar structure as a novel approach to inhibit growth and metastatic growth of different subtypes of breast cancers.

2. KEYWORDS:

Breast cancer, Malaria, treatment, metastasis, Recombinant protein, drug conjugate

3. OVERALL PROJECT SUMMARY:

In the past year of the project we have successfully produced recombinant VAR2CSA that binds with high affinity and high specificity to breast cancer cells and breast cancer tissue biopsies. We have demonstrated that the protein binds to distinct secondary modified proteoglycans exclusively present on breast cancer cells and that targeting these with recombinant VAR2CSA interferes with key functions of the cancer cell like growth, migration and invasion. There are no deviations to the agreed research plan and all major tasks has been initiated and some finalized (see below section for breakdown of results).

4. KEY RESEARCH ACCOMPLISHMENTS:

NOTE: The below reporting relates both to work done at BCCRC/ UBC in Dr. Daugaard's laboratories as well as the part done by Dr. Salanti in Copenhagen. Place of a given task is indicated in brackets.

RE: Major work task I (related to aim I):

A stable expression system for the production of recombinant VAR2CSA has been tested and validated in Shuffle E. coli cells. Most importantly we have developed a methods to ferment VAR2CSA in Shuffle cells, enabling us to produce sufficient amounts of recombinant protein for this project. **Figure 1** shows the result of one of the optimized fermentations resulting in a high yield pure protein for the subsequent cellular work. We are currently trying to move the expression system into insect cells and are in the process of mutating glycosylation sites in the VAR2CSA protein (as glycosylation of the protein inhibits its activity) (data not shown, in progress). The produced proteins has been tested for binding affinity and specificity to a panel of breast cancer cells, and we have shown that the prokaryotic produced proteins binds breast cancer cells with high affinity (1nM) and high specificity (**Figure 2 & 3**). Scale-up

batches (15 L fermentations) are currently being made for subsequent in vivo experiments. Accordingly, the work within major work task I is on schedule. (All this work has been done at UCPH/Salanti).

RE: Major work task II (related to aim II):
To conduct the work outlined in major work task II, we tested a number of different purification technologies including the TAP-TAG approach. It turned out that the best approach was not the TAP-TAG, but a column-based approach where recombinant VAR2CSA was immobilized within a purification column. Using this approach, we have until now successfully identified a number of proteoglycans that interact with the recombinant malaria protein VAR2CSA when sulfated on carbon 4 of the CS backbone. We have identified CSPG4 as a major protein in breast cancer cells, but also a range of new targets that needs to be validated. Using a holistic approach by overexpressing all human plasma membrane-associated proteins on the surface of HEK cells and probing with recombinant VAR2CSA we have identified 17 proteoglycans that can support VAR2CSA binding (by expressing a CSA chain), several of these were identified as being overexpressed in human breast cancer tissue (**Figure 4**). Accordingly, the work within major work task II is on schedule. (The method for pulling down 4 breast cancer associated proteoglycans was developed at UCPH/Salanti and performed by a PhD student from UCPH working in Daugaard's laboratory at UBC/VPC).

RE: Major work task III (related to aim III):
The work described in Subtask III-A has been finalized. We have with success investigated the internalization patterns of recombinant VAR2CSA and found that the protein internalize into intracellular structures within 15-20 minutes after addition to the tumor cell culture and that it ends up in the lysosomal compartment. (This work has been done both at UCPH and BCCRC) (**Figure 5**). The work described in Subtask III-B is well on its way and on schedule. We have identified the main signaling pathway affected by recombinant VAR2CSA (Integrin signaling) and validated this biochemically. We are currently investigating functional readouts of this signaling pathway. A PhD student enrolled at UCPH, working with Dr. Daugaard at VPC/UBC is doing this work.

RE: Major work task IV (related to aim IV):
The work described in Subtask IV-A, has been concluded according to plan. We have successfully established an IHC procedure for high-throughput tissue analysis on the VentureDiscovery platform. An important finding here is that the fixed tissue cannot be subjected antigen retrieval procedures (otherwise used for most IHC antibodies), as this process destroys the glycosaminoglycan target on the tissue. Based on this we have identified the optimal concentration of the recombinant VAR2CSA to be 500 pM in this assay. The optimized protocol is based on a primary incubation of a V5-tagged VAR2CSA, followed by a secondary incubation with a mouse monoclonal anti-V5 antibody, finalized by a detection step using anti mouse-HRP (**Figure 6 and 7**). The work described in Subtask IV-C, has commenced and we have tested the first test-TMA with encouraging results. The first indication is that we bind approximately 80-90% of the breast tumor specimens on the test array. (This work is done at UBC using protein from UCPH).

RE: Major work task V (related to aim V):
To facilitate coupling of saporin to VAR2CSA we have made a VAR2CSA protein with a biotin acceptor site and procured saporin coupled to streptavidin. The biotin-VAR2 drug conjugate kills a several tested breast cancer cells with nM IC50 values (This work has been done at UCPH and in vitro efficacy has been validated at UBC) (**Figure 5**). We are now proceeding with a new strategy for toxin conjugation based on melamide linkage. This work is ongoing.

RE: Major work task VI (related to aim VI):
The work outlined under Major work task VI has been initiated. As described in Subtask VI-A we are testing a panel of human breast cancer cell lines for their ability to bind recombinant VAR2CSA. To date we have tested 9 breast cancer cell lines and are planning to test the rest of our panel within the next 2-3 months. The result so far is very encouraging in the sense that all tested cell lines can be bound by recombinant VAR2CSA (data are being accumulated as lines are being tested, data not shown and **Figure 3**).

As described in Subtask VI-B, we are investigating the functional consequences of VAR2CSA binding to breast cancer cells. This work was recently initiated according to plan and our first results with the triple negative breast cancer cell line MDA-MB-231 suggests that recombinant VAR2CSA inhibits cell motility, but not proliferation, of these cells. Over the next 6 months we will include more cell lines in this analysis and hopefully be able to get a more detailed picture of this interesting phenotype (**Figure 8**).

5. CONCLUSION:
In summary the research is progressing according to the plan. We have managed to produce and quality control a scale up production of the malaria protein VAR2CSA. We have extensive data demonstrating that this protein specifically targets sulfated chondroitin sulfate A proteoglycans present on all tested breast cancer cells and the vast majority of tested tissue biopsies. Using pull down assays we have an overview of what the protein cores is and our functional in vitro assays demonstrate that targeting these proteoglycans interferes with integrin mediated focal adhesion. The next steps is to start up the animal experiments and show how IV injection of the VAR2CSA protein interferes with these pathways in vivo, in addition we have produced protein to test in vivo in subcutaneous models.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

Manuscripts:
Manuscript 1:
Salanti et al. Submitted to *Cancer Cell*.
Manuscript 2:
Clausen et al. Submission to *Molecular Cell* aimed for summer 2015.

Presentations:
A) 2013. Danish Cancer Society Research seminars; Glycosaminoglycans in cancer. Invited presentation. *Invited, Daugaard.*
B) 2013. Utilizing evolutionarily refined parasite-host anchor proteins to target malignant

disease. The 9th Tuscany Retreat on Cancer Research, Italy. *Invited, Daugaard.*
C) 2014. Gordon research conference, July 6-11; Targeting of cancer-specific chondroitin sulfate on circulating tumor cells using an evolutionary refined malaria protein. *Poster, Clausen.*
D) 2014. National Annual PhD meeting in Oncology, March 26-27: Development of new diagnostic
cancer methods using malaria proteins. *Invited, Salanti.*
E) (2014). Utilizing Plasmodium falciparum placental tropism to define a common oncofetal glycosaminoglycan signature. European Workshops on Cell Death (EWCD). Session: Novel Cancer Therapeutic strategies. *Invited, Daugaard.*
F) (2014). Malignancy-associated glycosamineoglycan Alterations in Urothelial Cancer. Leverage from other indications. 8th Annual Lorne Sullivan Research Day, Vancouver, Canada. *Invited, Daugaard.*
G) (2014). A Novel approach to broadly target human cancer based on a glycosaminoglycan-binding
malaria protein. Ludwig Institute Seminar Series. University of Oxford, Oxford, United Kingdom
Invited, Daugaard.
H) (2014). Transformed cells: exploring new targeting approaches. Medical University of Vienna, Vienna, Austria. *Invited, Daugaard.*

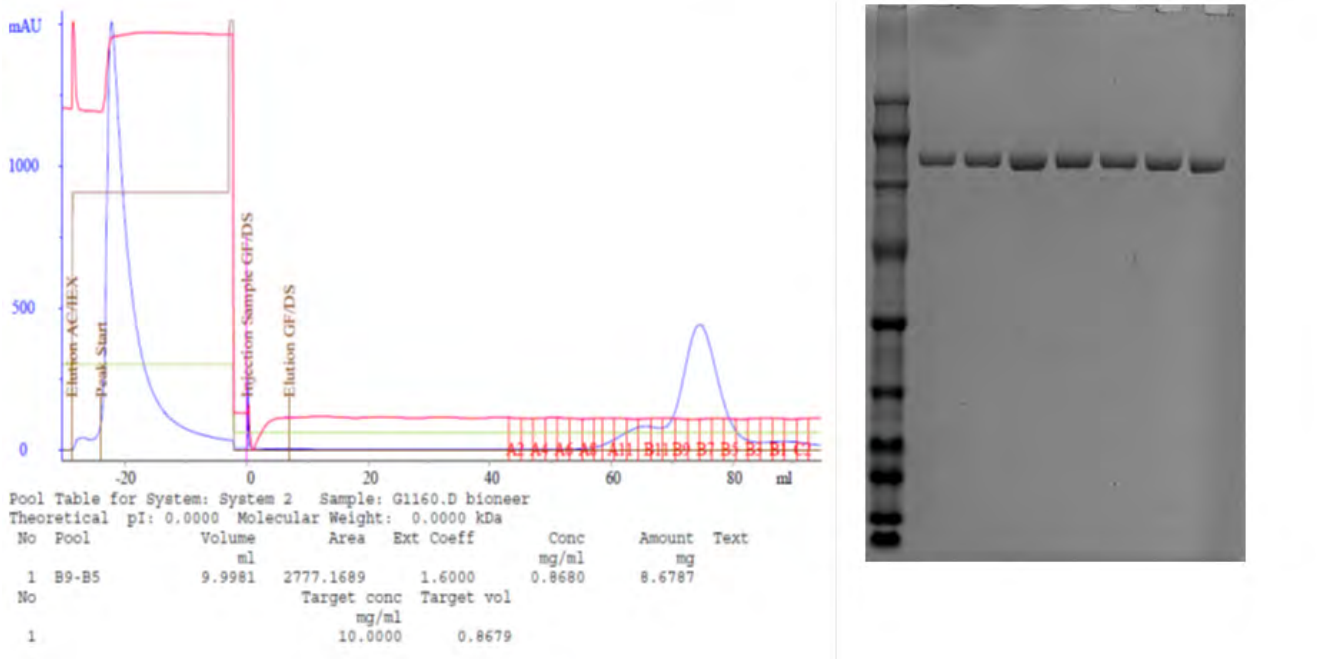
7. INVENTIONS, PATENTS AND LICENSES
Nothing to report

8. REPORTABLE OUTCOMES:
A) We have demonstrated that we can produce, under method compatible with GMP production, sufficient amounts and in high quality VAR2CSA to be the future treatment to breast cancer.
B) We have demonstrated that VAR2CSA binds to around 90% of all tested breast cancer biopsies.
C) We have demonstrated that VAR2CSA binds to CSA on proteoglycans that has been shown to be key to breast cancer progression, and that upon binding the ligand is internalized – increasing the feasibility of making a successful drug conjugate.

9. OTHER ACHIEVEMENTS:
Nothing to report

10. REFERENCES:
Nothing to report

11. APPENDICES:
Figure 1-8



Pool Table for System: System 2 Sample: G1160.D bioneer
Theoretical pI: 0.0000 Molecular Weight: 0.0000 kDa

No	Pool	Volume ml	Area	Ext Coeff	Conc mg/ml	Amount mg	Text
1	B9-B5	9.9981	2777.1689	1.6000	0.8680	8.6787	

No	Target conc mg/ml	Target vol
1	10.0000	0.8679

Figure 1. Example of the purification of a single fermantation experiment of VAR2CSA in E. coli Shuffle cells. After a two step purification (Affinity chromatography and size exclusion) the yield is around 15mg/L culture. The left panel shows the size exclusion profile, demonstrating that the protein express predominantly as a monomer with no aggregates. The right panel is an SDS page showing the monomer peak, demonstrating a very high purity of the protein

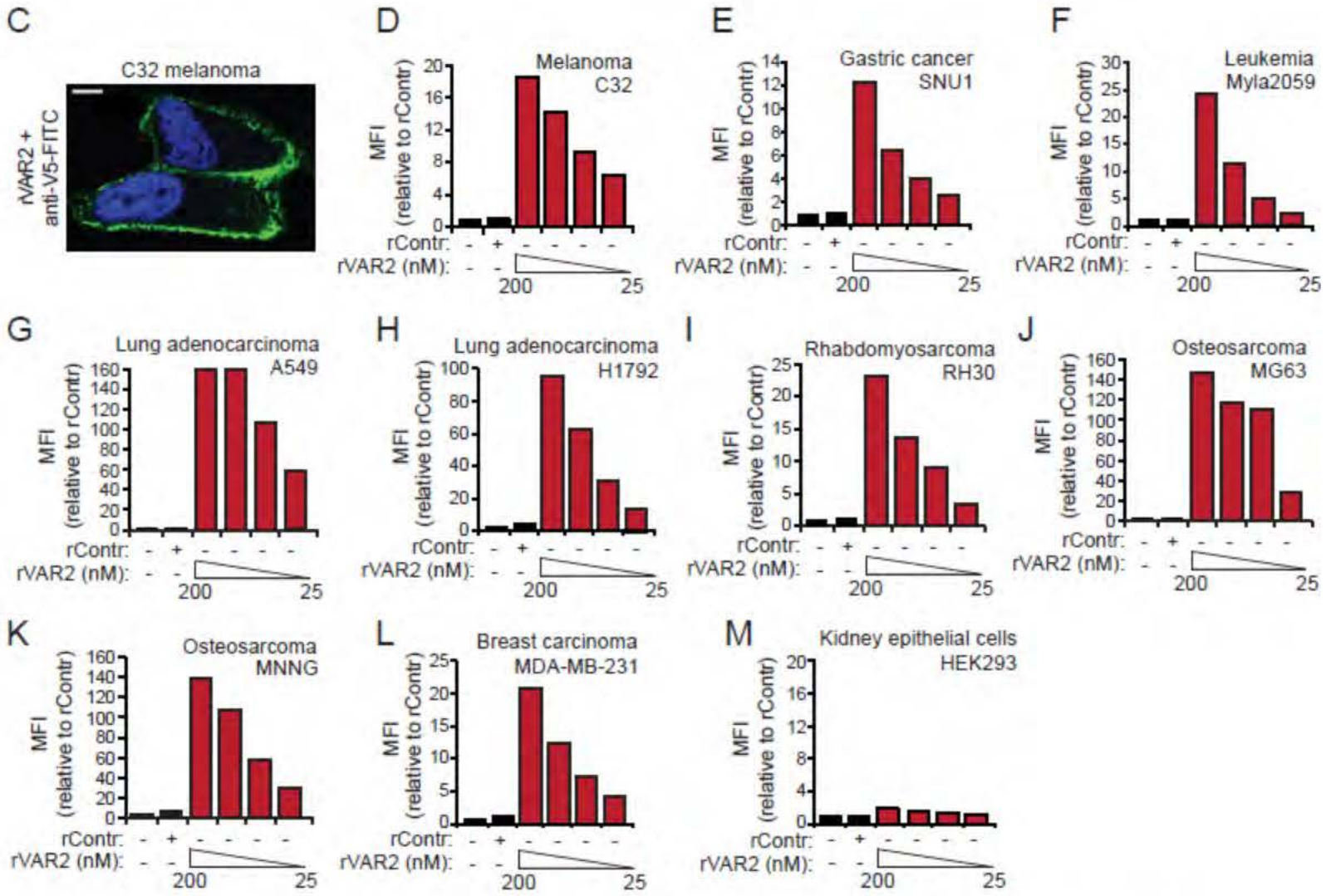


Figure 2. Testing of purified recombinant VAR2CSA binding to panels of cancer cells, including MDA-MB-231 (panel L). VAR2CSA binds to all tested cancer cells measured by flow cytometry using a gradient concentration. No binding was observed to control HEK cells. An example of the staining pattern is shown in panel C.

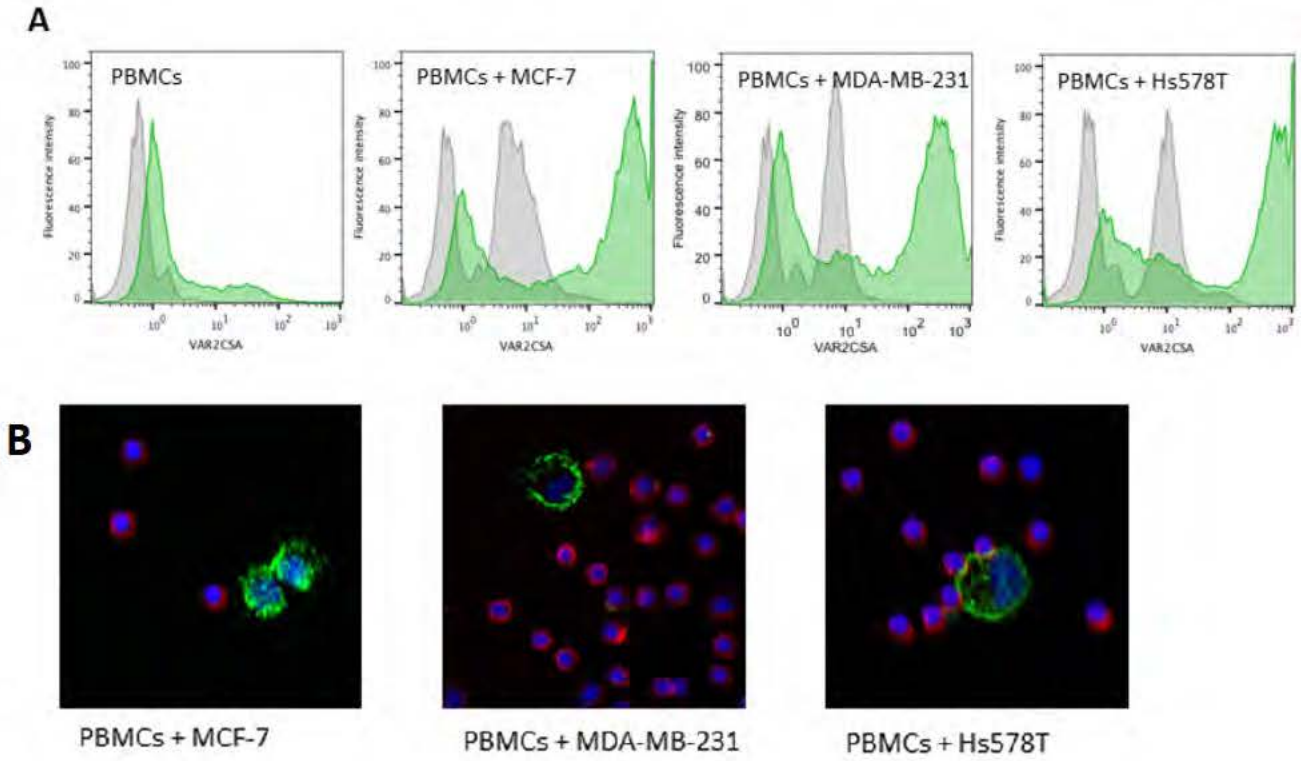


Figure 3. A) Following purification of mononuclear cells of the peripheral blood (PBMCs) a panel of breast cancer cell lines were spiked into the healthy PBMCs in a 1:1 ratio and VAR2CSA binding was detected using flow cytometry. VAR2CSA showed no binding to the PBMCs whereas it bound to the MCF-7, MDA-MB-231 and Hs578T cell lines, confirming the cancer specificity. **B)** Breast cancer cells were spiked into PBMCs in a 1:10.000 ratio and VAR2CSA staining (green) was detected using the automatic scanning system of the CytoTrack platform. Specificity was confirmed by cell morphology, DAPI positivity (blue) and CD45 negativity (red). **C)** Flow cytomety binding of recombinant VAR2CSA to four different breast cancer cell lines show that all cell lines binds VAR2CSA, to varying degrees.

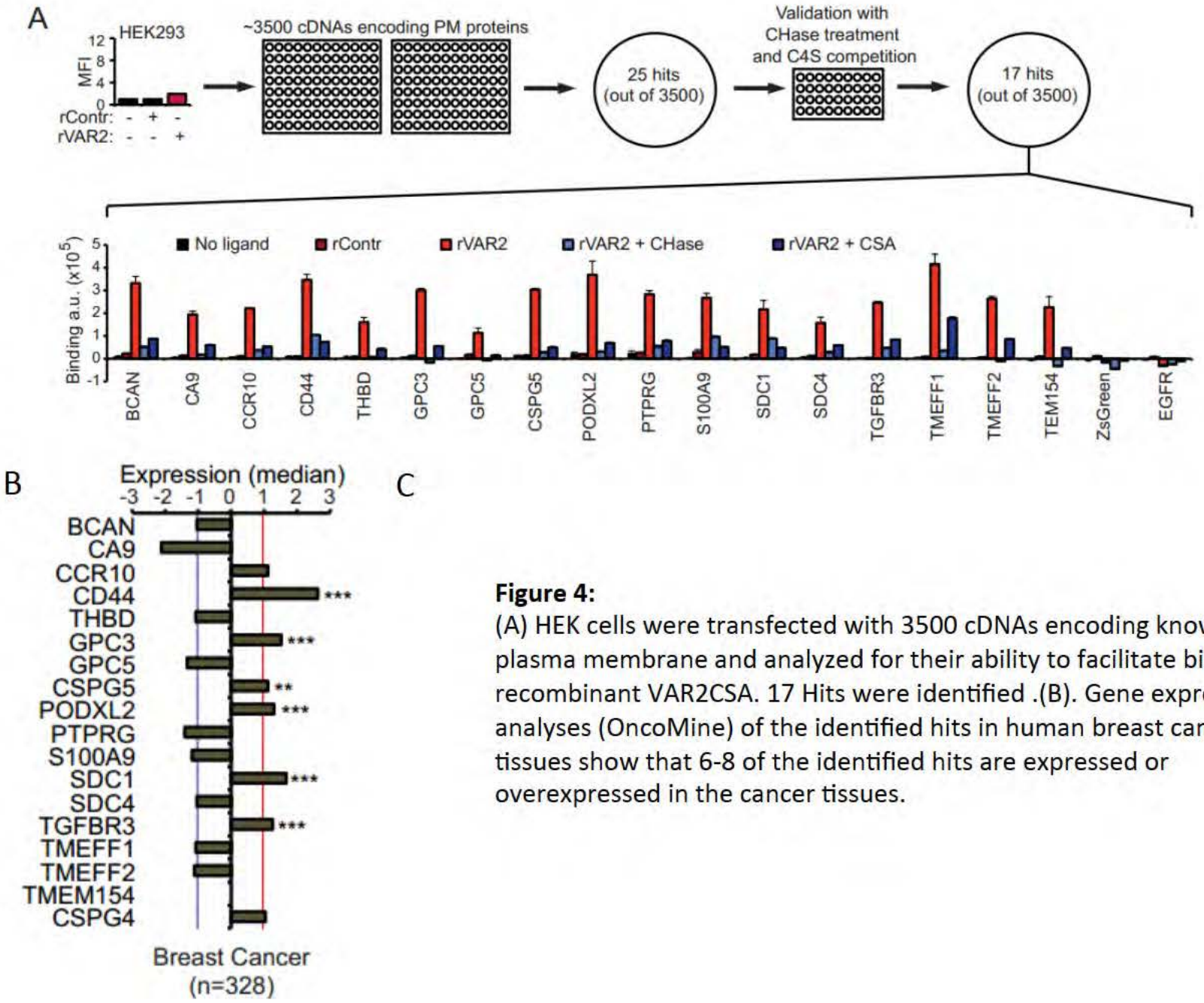


Figure 4:
(A) HEK cells were transfected with 3500 cDNAs encoding known plasma membrane and analyzed for their ability to facilitate binding to recombinant VAR2CSA. 17 Hits were identified .(B). Gene expression analyses (OncoMine) of the identified hits in human breast cancer tissues show that 6-8 of the identified hits are expressed or overexpressed in the cancer tissues.

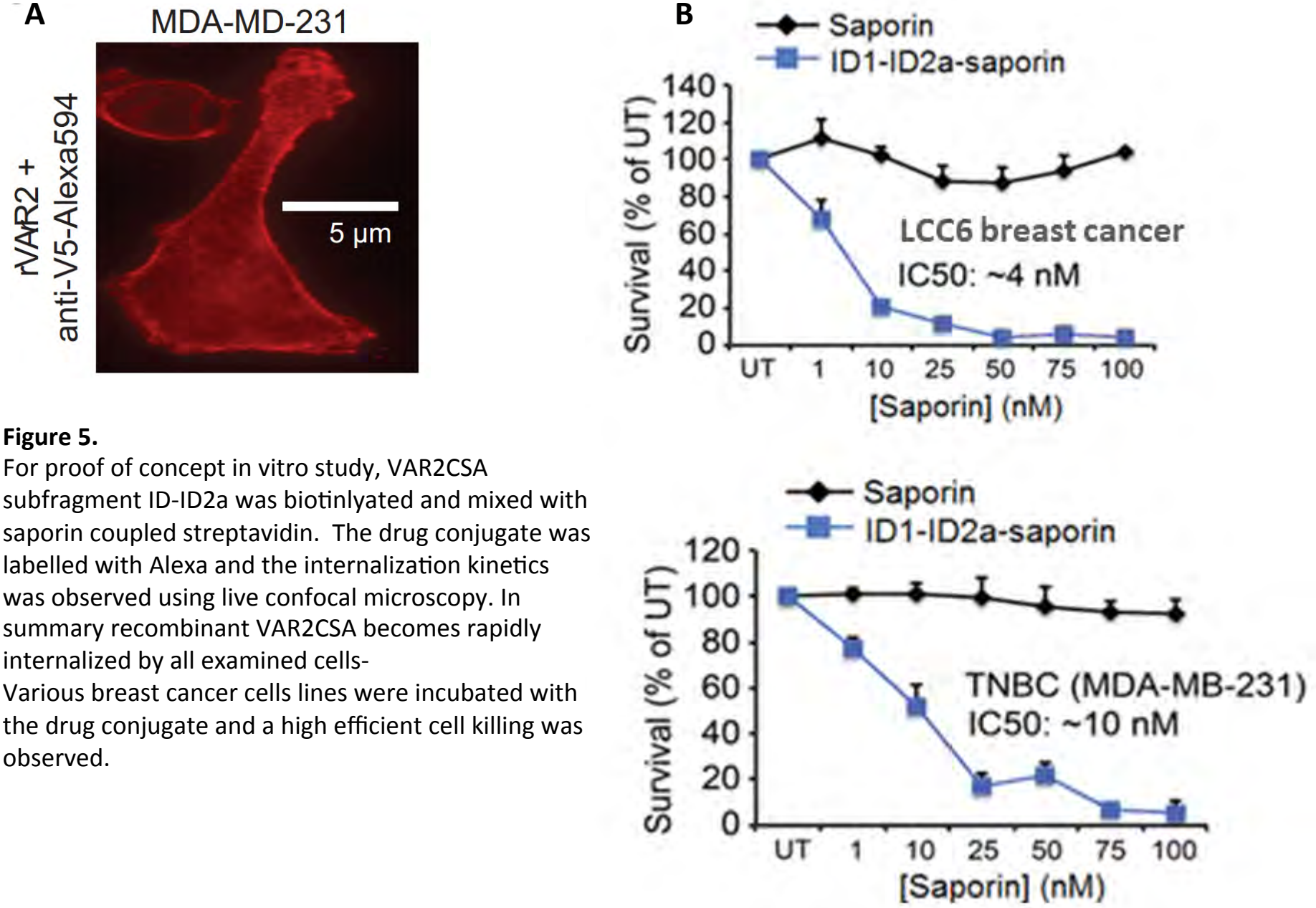


Figure 5.
For proof of concept in vitro study, VAR2CSA subfragment ID-ID2a was biotinlyated and mixed with saporin coupled streptavidin. The drug conjugate was labelled with Alexa and the internalization kinetics was observed using live confocal microscopy. In summary recombinant VAR2CSA becomes rapidly internalized by all examined cells-
Various breast cancer cells lines were incubated with the drug conjugate and a high efficient cell killing was observed.

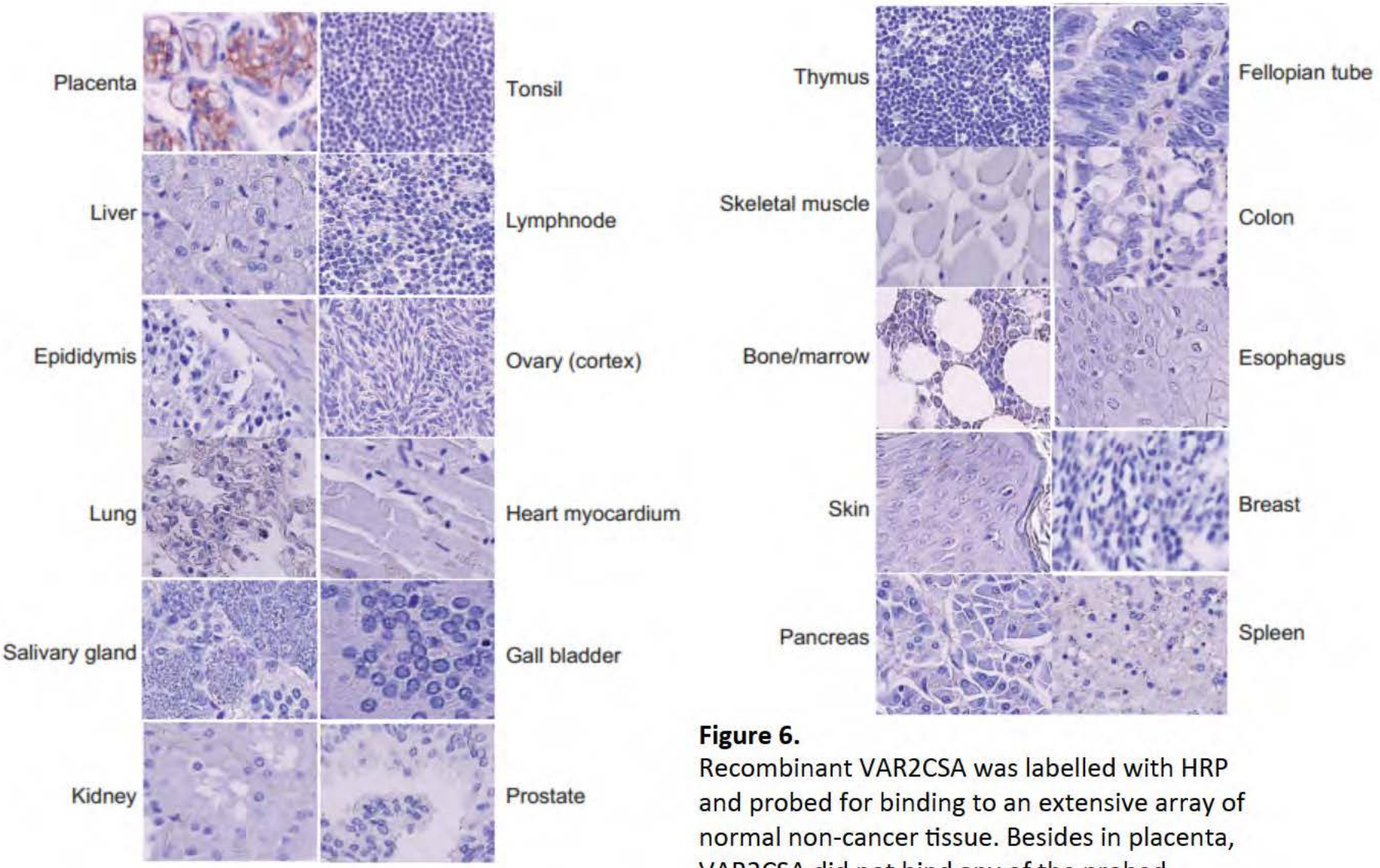
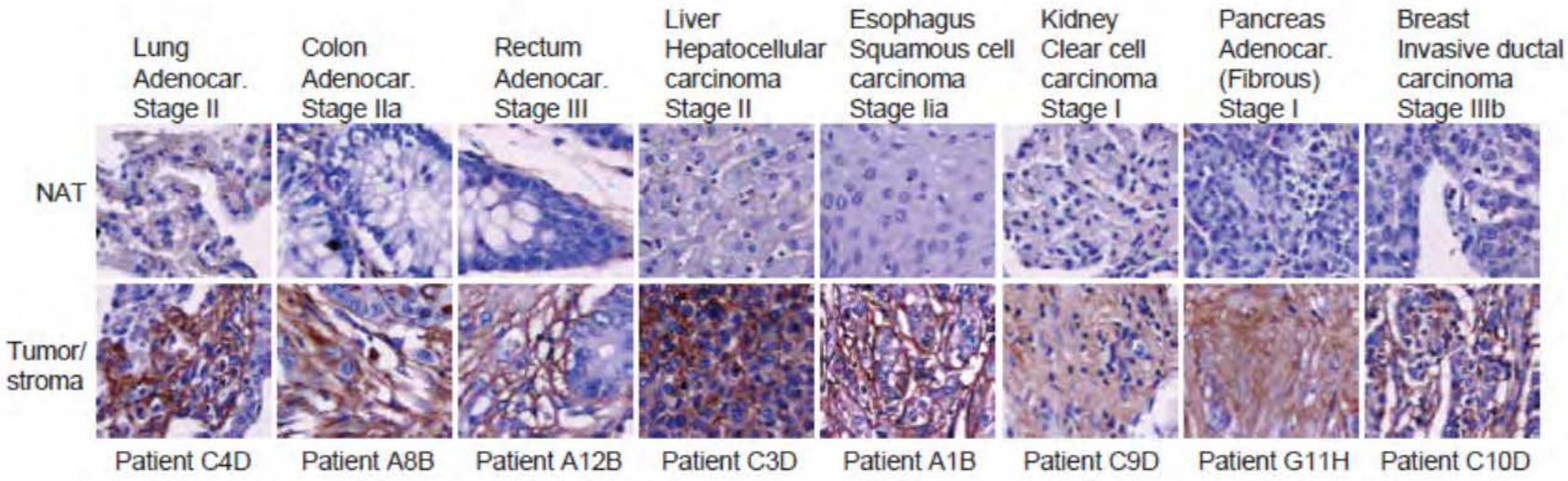


Figure 6.
Recombinant VAR2CSA was labelled with HRP and probed for binding to an extensive array of normal non-cancer tissue. Besides in placenta, VAR2CSA did not bind any of the probed tissues



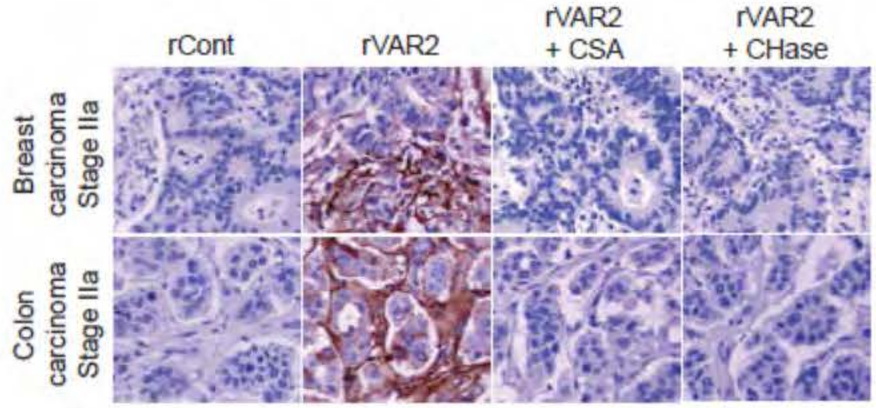


Figure 7.
Recombinant VAR2CSA was labelled with HRP and probed for binding to an extensive array of human cancer tissue and matched adjacent normal tissue. VAR2CSA binds all tested cancer tissues, with no observed binding to normal tissue (upper panel).
Competition with soluble CSA or chondroitinase treatment of the tissue removes the VAR2CSA binding (lower panel

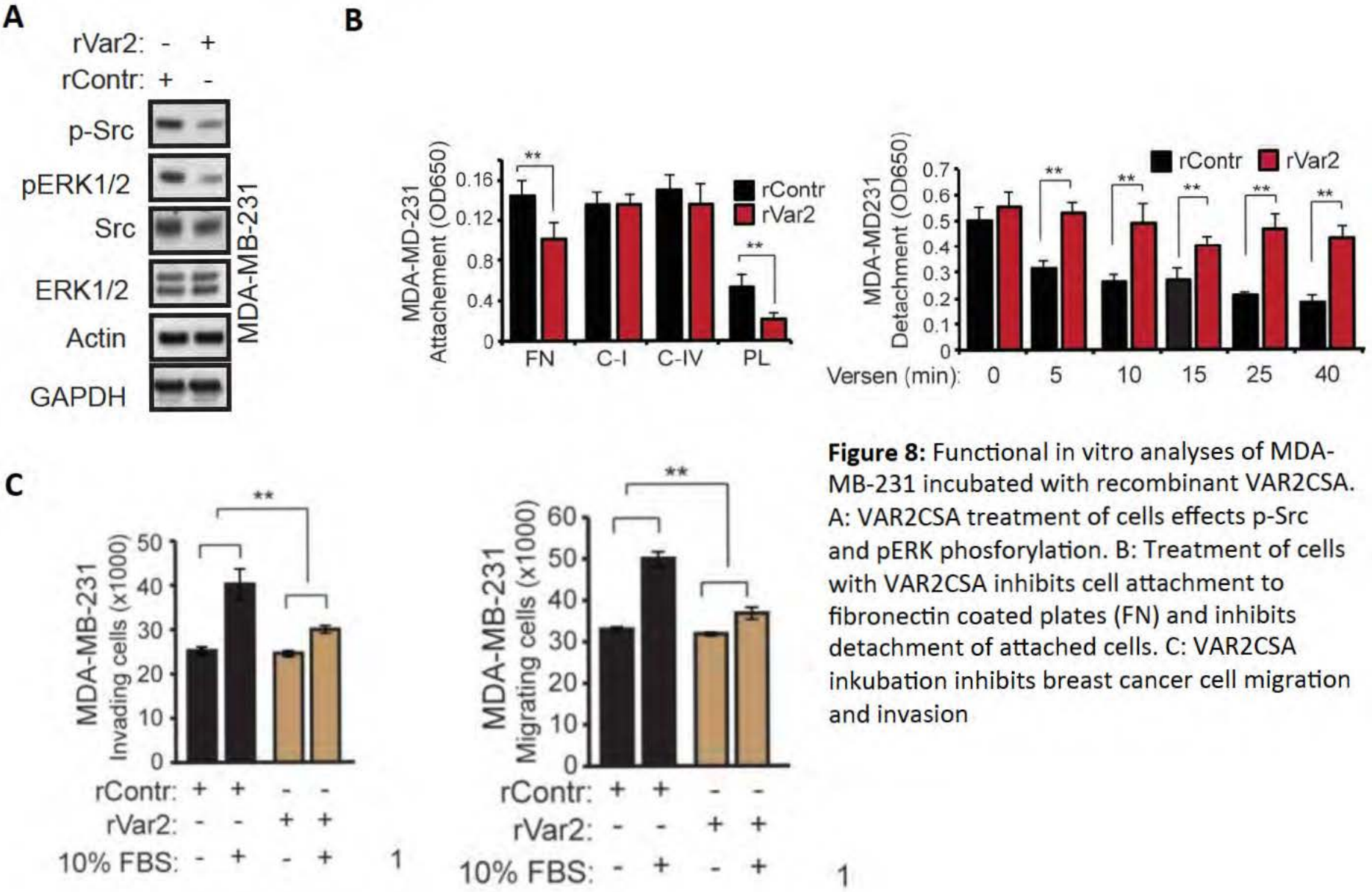


Figure 8: Functional in vitro analyses of MDA-MB-231 incubated with recombinant VAR2CSA. A: VAR2CSA treatment of cells effects p-Src and pERK phosforylation. B: Treatment of cells with VAR2CSA inhibits cell attachment to fibronectin coated plates (FN) and inhibits detachment of attached cells. C: VAR2CSA inkubation inhibits breast cancer cell migration and invasion